Interactions of polynucleotides and their components

IV. Acid denaturation of polyadenylic-polyuridylic complexes*

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Interactions between A and U and between A and A were studied by spectrophotometric titrations in the acid pH branch. Titrations of the 1 A−1 U solutions have shown that, at higher ionic strengths ($\omega > 0.1$), there occurs first a conformational transition of the (A+U) form to the (A+U+U) form in the pH range 5.2 to 3.8: only at lower pH one has the dissociation of (A+U+U) to the components. A phase diagram, showing the stability conditions of the different A−U structures as a function of ionic strength and pH was drawn.

By comparing the alkaline and acid dissociations of 1 A−1 U and 1 A−2 U solutions, the pK values of A can be determined: several expressions for this determination can be proposed depending on the extent to which the cooperative dissociation of (A+U) is influenced by the cooperative formation of (A+A) from A. The results suggest that, in contrast to U, the polyelectrolyte character of the polynucleotide, originating from the phosphate backbone, cannot account for the pK shift of the adenine residue of A. A knowledge of the dissociation constant of the adenine residue is useful in calculating the interaction energy of (A+A). A undergoes a sharp conformational change on titration with acid in the pH range 6.1 to 5.6, which has been interpreted as a A−A interaction with formation of a (A+A) helical structure. By comparing the transition pH of (A+A) with the dissociation constant of the adenine residue, it is possible to calculate the free energy change of (A+A) formation by use of the relation $\Delta F = -2{,}3\, R\, T\, \Delta p\, K$. A qualitative comparison of the determined ΔF values with the calorimetric enthalpy changes suggests that A−A interactions are characterised by small entropy changes.

The studies of thermal, alkaline and acid denaturation of natural and synthetic polynucleotides have yielded useful information about the nature and stability of nucleic acid interactions [1–7]. From spectrophotometric studies of A−U solutions at several ionic strengths, temperatures and relative proportions of A and U, the stability conditions of the various A−U structures have been determined [8]. Furthermore the alkaline dissociation of A−U complexes has been analysed quantitatively in order to get information about the thermodynamic basis of these interactions [1–3, 9]. The acid dissociation has so far only yielded qualitative data about the stability zones of various A−U complexes in the presence of (A+A) [4]. In the present investigation, we have drawn a parallel between the alkaline and acid denaturation of A−U complexes in order to obtain quantitative results about the pK values of A from the acid denaturation.

Experimental

The materials that have been used and the spectrophotometric titration method have been described previously [10].

* Abbreviations: A: polyriboadenylic acid; U: polyribouridylic acid; C: polyribocytidylic acid; xA−yU: a solution containing x equivalents A and y equivalents U; A−U: solutions of (or complexes between) A and U without stoichiometric or structural specifications; (A+U): the two-stranded complex of the homopolymers A and U in a proportion 1:1; (A+U+U): the three-stranded structure containing one A and two U chains; (A+A): the two-stranded aggregate containing two A chains; (C+C): the two-stranded aggregate of two C chains; ω: ionic strength = molar concentration of NaCl.

[1] R. C. WARNER, J. biol. Chemistry **229**, 711 [1957].
[2] R. C. WARNER and E. BRESLOW, Proc. Fourth Int. Congr. Biochem. **Vol. 9**, Pergamon Press, New York 1958, p. 157.
[3] R. C. WARNER, in: Informational Macromolecules, H. J. VOGEL, V. BRYSON, and J. O. LAMPEN, Eds., Academic Press, New York 1963, p. 146.
[4] R. A. Cox, Biochim. biophysica Acta [Amsterdam] **68**, 401 [1963].
[5] R. A. Cox and U. Z. LITTAUER, Biochim. biophysica Acta [Amsterdam] **72**, 188 [1963].
[6] R. A. Cox, Biochim. biophysica Acta [Amsterdam] **72**, 203 [1963].
[7] U. Z. LITTAUER and H. EISENBERG, Biochim. biophysica Acta [Amsterdam] **32**, 320 [1959].
[8] CH. L. STEVENS and G. FELSENFELD, Biopolymers **2**, 293 [1964].
[9] A. LITAN, J. physic. Chem. **70**, 3107 [1966].
[10] J. CLAUWAERT, J. STOCKX, and L. VANDENDRIESSCHE, Z. Naturforschg. **23 b**, 33 [1968].

Results

The alkaline branch of the spectrophotometric titration curves of A – U complexes may be compared directly with the alkaline dissociation branch of U from which the free energy change of A – U aggregation can be determined [1–3, 9, 10] (fig. 1). The acid titration curve is quite intricate and not so readily interpretable because dissociation of the A – U complexes is accompanied and perhaps influenced by (A + A) formation.

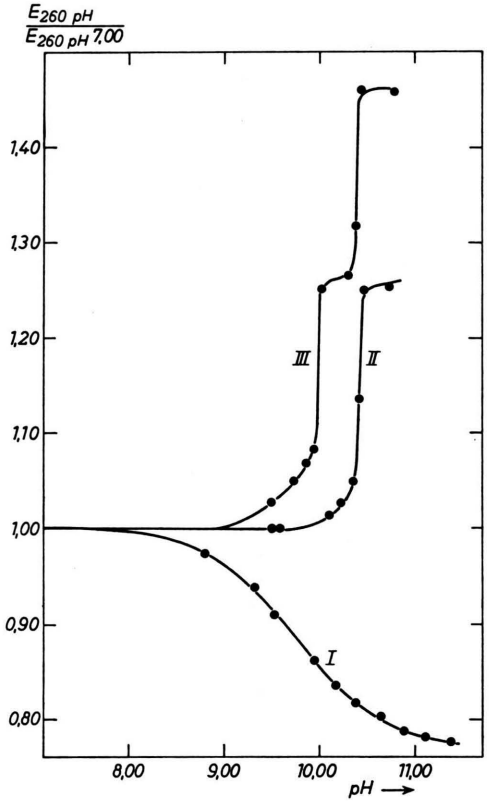

Fig. 1. Spectrophotometric titration curves at $\omega = 0.3$ to alkaline pH values of 1 U (curve 1), a 1 A–1 U (II) and a 1 A–2 U (III) solution.

The results about the acid dissociation of A – U complexes are given in fig. 2. The dissociation curve of the 1 A – 1 U solution at $\omega = 0.3$ is typical for all acid dissociation curves at ionic strengths higher than 0.1. From spectral changes in the pH range 5.0 to 4.0, it can be deduced that we have first a structural transition which cannot be explained as a dissociation of (A + U) to the component polynucleotides. In order to get more information about the nature of this structural change, spectrophotometric

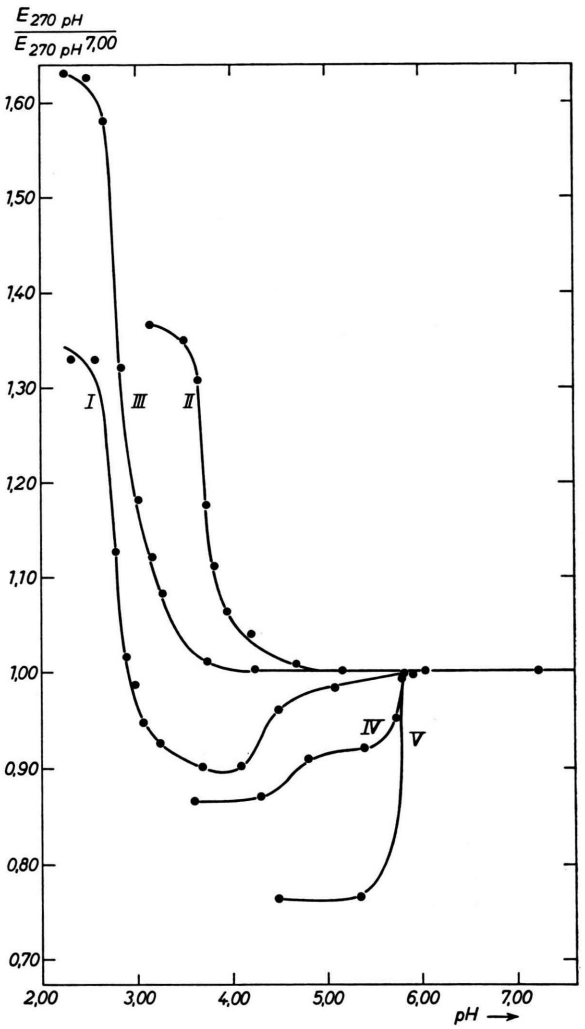

Fig. 2. Spectrophotometric titration curves to acid pH values of 1 A–1 U (curve I), 1 A–2 U (III), 1 A (V) and 2 A–1 U (IV) solution at $\omega = 0.3$ and of a 1 A+1 U solution at $\omega = 0.06$ (II).

titrations to acid pH values have been performed at $\omega = 0.3$ of 1 A – 1 U, 1 A – 2 U, 2 A – 1 U and A solutions and at $\omega = 0.06$ of a 1 A – 1 U solution. Several conclusions are obvious. The structural change found in the pH range 5.0 to 4.0 only occurs for 1 A – 1 U solutions of higher ionic strengths; the 1 A – 2 U solutions lack it in this pH range but dissociate to the component polynucleotides at the same pH as the 1 A – 1 U solutions; a 1 A – 1 U solution of lower ionic strength ($\omega < 0.1$) also lacks that specific spectral change. There is a complete analogy between acid dissociation and thermal dissociation of 1 A – 1 U solutions [8]. When preparing

1 A – 1 U solutions of moderate and higher ionic strengths at room temperature, a few percent of poly A and poly U is complexed into (A + U + U)[12] so that in a 1 A – 1 U solution of moderate or high ionic strength we always deal with the presence of a small amount of free poly A; this leaves the possibility that the spectral changes in the pH range 5.0 to 4.0 may be due to (A + A) formation from free poly A. The titration curves of A and 2 A – 1 U solutions invalidate this supposition: a A solution shows a spectral change at higher pH which is due to a (A + A) formation[13–15]; a 2 A – 1 U solution, containing (A + U) and an equivalent quantity of free A, shows two transitions: at a higher pH the free A interacts to form (A + A), the remaining (A + U) showing the same structural change as a 1 A – 1 U solution.

These findings suggest that the spectral changes of the 1 A – 1 U solutions of higher ionic strengths in the pH range 5.0 to 4.0 originate from the transition $2(A+U) \rightarrow (A+U+U) + {}^1/_2(A+A)$. At pH 7.0 and ionic strength 0.3 mixing curves of 1 A – 1 U solutions with an equivalent of U confirmed this by showing strong hypochromicity and we obtained the spectrum of (A + U + U); mixing of 1 A – 2 U with an equivalent of U yielded no spectral changes. At pH 5.0, the same results have been obtained. When mixing at pH 3.8 a 1 A – 1 U solution with an equivalent U, no spectral changes are observed: this confirms the hypothesis that, starting from a 1 A – 1 U solution, the transition $2(A+U) \rightarrow (A+U+U) + {}^1/_2(A+A)$ occurs when the pH is lowered to 4.0.

The spectral changes arising by mixing poly A and poly U in several proportions and by heating 1 A – 1 U and 1 A – 2 U solutions made it possible to select several appropriate wavelengths for studying the interactions $A + U \rightarrow (A+U)$ or $(A+U) + U \rightarrow (A+U+U)$ [8, 12]. They cannot be used in our study of acid dissociation of A + U mixtures because we probably also deal with the interaction

$$2(A+U) \rightarrow (A+U+U) + A \rightarrow (A+U+U) + {}^1/_2(A+A).$$

The spectral changes due to the interaction $A \rightarrow {}^1/_2(A+A)$ compete with those due to the transition $2(A+U) \rightarrow (A+U+U) + A$ and the individual contributions cannot be determined with sufficient accuracy.

The results of the acid denaturation of A – U solutions at 20 °C and several ionic strengths are summarised in table 1. We have the transitions

$$2(A+U) \rightarrow (A+U+U) + {}^1/_2(A+A) \text{ at } pH_0,$$
$$(A+U) \rightarrow {}^1/_2(A+A) + U \text{ at } pH_{s1} \text{ and}$$
$$(A+U+U) \rightarrow {}^1/_2(A+A) + 2\,U \text{ at } pH_{s2}.$$

In order to deduce the pK of A from the acid dissociation studies of A – U complexes, we needed information about (A + A) formation. A is known to undergo a structural transition in aqueous solution as a function of pH: at neutral pH it is believed to be a flexible one-stranded helical structure with stacked bases and lowering the pH would result into the formation of (A + A)[16–20]. From potentiometric and spectrophotometric titration studies a two step reaction scheme has been proposed: at first protonation of a fraction of A takes place: $A + H^{\oplus} \rightarrow AH^{\oplus}$; following this a helical structure is formed between a protonated and a neutral A chain: $AH^{\oplus} + A \rightarrow (A + AH^{\oplus})$. The ionic strength influences the transition pH of the single stranded to the double stranded structure: an increase in ionic strength results into a displacement of the transition pH to lower values. Table 2 gives the midpoint pH values of the transition $A + A \xrightarrow{H^{\oplus}} (A + AH^{\oplus})$ at 20 °C in function of ionic strength.

Discussion

Acid dissociation of A – U complexes

It has been shown that in dilute solutions a 1:1 mixture of A and U always leads to the formation of (A + U) at room temperature; by raising the temperature of a 1 A – 1 U solution at higher ionic strengths, (A + U) is converted to (A + U + U) and A. From this a phase diagram has been drawn

[11] A. Katchalsky and A. Oplatka, Israël J. med. Sci. **2**, 4 [1966].
[12] R. D. Blake and J. R. Fresco, J. molecular Biol. **19**, 145 [1966].
[13] J. R. Fresco and P. Doty, J. Amer. chem. Soc. **79**, 3928 [1957].
[14] P. O. P. Ts'o, G. K. Helmkamp, and C. Sander, Proc. nat. Acad. Sci. USA **48**, 686 [1962].
[15] D. N. Holcomb and I. Tinoco, Biopolymers **3**, 121 [1965].
[16] J. R. Fresco, Tetrahedron [London] **13**, 185 [1961].
[17] A. Rich, D. R. Davies, F. H. C. Crick, and J. D. Watson, J. molecular Biol. **3**, 71 [1961].
[18] J. Brahms, J. Amer. chem. Soc. **85**, 3298 [1963].
[19] V. Luzzati, A. Mathis, F. Masson, and J. Witz, J. molecular Biol. **10**, 28 [1964].
[20] J. Applequist and V. Damle, J. Amer. chem. Soc. **88**, 3895 [1966].

showing the stability conditions of the various A – U structures in function of ionic strength and temperature starting from a 1 A – 1 U solution [8]. It has been suggested that the free energy change associated with the formation of the single stranded ordered structure of A causes the conversion of the double stranded (A + U) to the three stranded (A + U + U) structure [8].

The spectrophotometric titrations to acid pH values of A – U solutions revealed similar transitions. A lowering of the pH of a 1 A – 1 U solution, containing the (A + U) complex, at higher ionic strength and at room temperature promotes the transition $2(A+U) \rightarrow (A+U+U) + A \rightarrow (A+U+U) + {}^1/_2(A+A)$.

The free energy change of the interaction $A \rightarrow {}^1/_2(A+A)$ is probably the driving force in the conversion of (A + U) to (A + U + U). Here too a phase diagram can be drawn which deliminates stability conditions of the various A – U complexes in function of pH and ionic strength (fig. 3). The labilisation of A – U structures either by heating

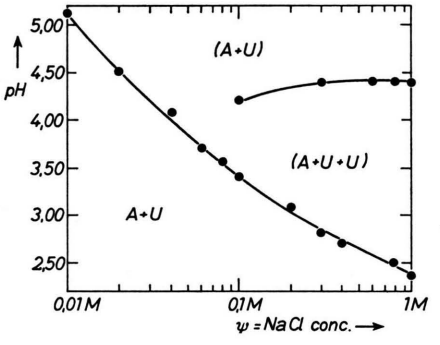

Fig. 3. "Phase diagram" of a 1 A–1 U solution at 20 °C in function of ionic strength and pH; (A+U): stability zone of the (A+U) complex; (A+U+U): stability zone of the (A+U+U) complex; A+U: conditions of pH and ionic strength where the polynucleotides do not interact.

the solution or by lowering the pH of the solution follows a parallel course. (A + U) breaks down at lower ionic strengths to the component polynucleotides in these conditions. At higher ionic strengths, where (A + U + U) is stable, the labilised (A + U) system regains stability by its transition to (A + U + U): the free energy change involved in $A \rightarrow {}^1/_2(A + A)$ perhaps influences the transition of the two – to the three stranded structure. When (A + U) is labilised by an increase in pH, the dissociation of the $HN_{(1)} - C_{(6)} = O$ groups of U makes the reorganisation of the labilised two stranded into the three stranded structure impossible: accordingly, the study of the stability vs. alkaline dissociation of A – U structures is little troublesome and offers a most reliable tool to track stability conditions and the energetic basis for the formation of A – U structures [9, 10].

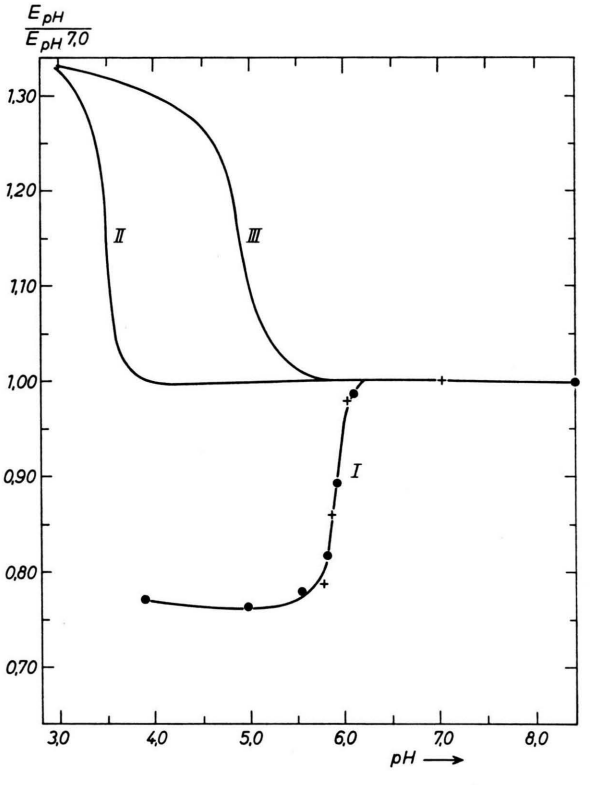

Fig. 4. Spectrophotometric titration curves of 1 A and 1 A–1 U solutions at $\omega = 0.1$ and 20 °C to acid pH values and back titration curves; I: forward and back titration curve of 1 A solution; II: forward titration curve of a 1 A–1 U solution and III: back titration curve of this 1 A–1 U solution.

pK values of A

It was our intention to determine the pK value of the adenine residue of A. As a first approach to the problem, we may suppose that adenosine, incorporated into A, undergoes a similar influence from the polynucleotide backbone as uridine incorporated in U: this supposition is justified in so far as the ribose-phosphate backbone of the polynucleotide solely determines the electrostatic potential of the macromolecule. For U it has been shown that this is

broadly the real situation [21, 22] but for A a base stacking has been accepted generally [19, 20, 23, 24] that may markedly influence the electrostatic potential of the polynucleotide. In spite of this, we can get a first approximation of the pK of A. Table 3 gives the pK values of A assuming that

$$pK\,\mathrm{A} - pK\,\text{adenosine} = pK\,\mathrm{U} - pK\,\text{uridine}.$$

This assumption is in keeping with the view that the phosphate backbone alone would determine the electrostatic potential of A.

The acid dissociation of A – U complexes may thus be used to determine the pK values of A. Mixing equivalent quantities of A and U at neutral pH yields (A + U).

At this pH the free energy changes by ΔF_0 when equivalent quantities of A and U are allowed to form (A + U). At lower pH values the NH_2 groups of A contributing to (A + U) are not protonable in their normal range: (A + U) should first dissociate before A can be protonated and the free energy of the (A + U) system increases by $\Delta F = \Delta F_0 + \Delta F_{\text{prot}}$ where ΔF_{prot} represents the free energy increase of a protonable group, with dissociation constant K, that is kept unprotonated up to a hydrogen ion activity h.

$$\Delta F_{\text{prot}} = 2.3\,RT \log \frac{\mathrm{K}+h}{K} = 2.3\,RT\,(pK - pH).$$

In the range of acid dissociation $(\mathrm{A}+\mathrm{U}) \overset{H^{\oplus}}{\underset{pH_s}{\rightleftarrows}} \mathrm{A}+\mathrm{U}$, it is required that

$$\Delta F = \Delta F_0 + \Delta F_{\text{prot}} = 0 \quad \text{or} \quad \Delta F_0 = 2.3\,(pH_s - pK).$$

Knowing ΔF_0 and pH_s, the pK can be determined. The ΔF_0 values of the interaction $\mathrm{A}+\mathrm{U} \rightarrow (\mathrm{A}+\mathrm{U})$ or $(\mathrm{A}+\mathrm{U})+\mathrm{U} \rightarrow (\mathrm{A}+\mathrm{U}+\mathrm{U})$ have been determined from spectrophotometric titrations of 1 A – 1 U and 1 A – 2 U solutions towards alkaline pH values. So we suppose: pH of alkaline dissociation – pK of U = pK of A – pH of acid dissociation of (A + U).

For (A + U + U), analogous relations can be deduced.

By labilisation of (A + U) or (A + U + U) by lowering the pH a small amount of A is set free and complexes at once to (A + A): the cooperative formation of (A + A) may thus influence the cooperative dissociation of (A + U). We have an experimental indication that this does happen indeed. The acid titration curves of A solutions are fully reversible. Starting from a neutral solution and lowering the pH we find at pH_{A+A} the transition $\mathrm{A}+\mathrm{A} \overset{50\%}{\rightarrow} (\mathrm{A}+\mathrm{A})$. Starting from a acid solution and increasing the pH we observe the transition $(\mathrm{A}+\mathrm{A}) \overset{50\%}{\rightarrow} \mathrm{A}+\mathrm{A}$ at the same pH. The acid spectrophotometric titrations of (A + U) solutions are not reversible however [4]; at pH_s we have the transition $(\mathrm{A}+\mathrm{U}) \rightarrow \mathrm{A}+\mathrm{U} \rightarrow \mathrm{U}+{}^1/_2(\mathrm{A}+\mathrm{A})$. Starting from an acid solution a hysteresis is observed since the dissociation of (A + A) must precede the formation of (A + U). We have ${}^1/_2(\mathrm{A}+\mathrm{A})+\mathrm{U} \rightarrow \mathrm{A}+\mathrm{U} \rightarrow (\mathrm{A}+\mathrm{U})$.

When the dissociation of (A + A) is not influenced by (A + U), formed from the free A and U of the solution, the back titration curve of the 1 A – 1 U solution has to show the dissociation of (A + A) at the same pH, pH_{A+A}, as the solution containing only A. When the dissociation of (A + A) is influenced by (A + U), the dissociation of (A + A) in a 1 A – 1 U solution has to take place at another, probably lower, pH. Spectrophotometric forward and back titration curves of A solutions in the acid region are reversible. The back titration curves of 1 A – 1 U solutions show a hysteresis due to (A + A) formation at lower pH values, but (A + A) in the presence of free U, dissociates at a lower pH than a pure (A + A) solution, thus indicating that, starting from an acid 1 A – 1 U solution, the formation of (A + U) accelerates the dissociation of (A + A). From this we may conclude as well that the dissociation of (A + U) by lowering the pH of a 1 A – 1 U solution will be influenced by (A + A) formation.

We assume that in the aggregation of poly A, set free from (A + U) at lower pH, to (A + A) the same free energy change ΔF is involved as in the formation of (A + A) from a neutral solution of A by lowering the pH. The free energy change of (A + A) formation can be determined from the following relations: at neutrality we have a single stranded A chain; when (A + A) formation occurs at the pK of A we have the free energy change

[21] J. Clauwaert and J. Stockx, Z. Naturforschg. **23 b**, 25 [1968].
[22] A. M. Michelson and C. Monny, Proc. nat. Acad. Sci. USA **56**, 1528 [1966].
[23] J. Brahms, Nature [London] **202**, 797 [1964].
[24] J. Brahms and W. F. Mommaerts, J. molecular Biol. **10**, 73 [1964].

$\Delta F_A = \Delta F_0$; when (A + A) formation occurs at higher pH, a fraction of the free energy change has to be used to protonate half the A at a pH where it normally shows little or no protonation:

$\Delta F_A = \Delta F_0 + \Delta F_{prot}$ where $\Delta F_{prot} = 2.3\ RT\,(pH - pK)$.

Starting from a neutral solution of A we have at the transition pH, pH_{A+A}:

$A + A \xrightarrow{50\%} (A + A)$ so that

$$\Delta F_A = \Delta F_0 + \Delta F_{prot} = 0 \text{ and}$$
$$\Delta F_0 = -2.3\ RT\,(pH_{A+A} - pK).$$

The free energy change of the interaction $A + A \rightarrow (A + A)$ occurring at a pH $\gg$ pK is given by

$$\Delta F_A = 2.3\ RT\,(pK - pH_{A+A}) + 2.3\ RT\,(pH - pK)$$
$$\Delta F_A = 2.3\ RT\,(pH - pH_{A+A}).$$

At pH < pK we assume that $\Delta F_A = 2.3\ RT\,(pK - pH_{A+A})$. While (A + U) is dissociating we have

$A + U) \rightarrow (A + U)$ $\quad \Delta F_1$,
$A + A \rightarrow (A + A)$ $\quad \Delta F_A$,
$(A + U) \rightarrow A + U \rightarrow {}^1/_2 (A + A) + U$ $\quad \Delta F = {}^1/_2 \Delta F_A - \Delta F_1$.

For the dissociation of (A + U + U) we have

$A + U \rightarrow (A + U)$ $\quad \Delta F_1$
$(A + U) + U \rightarrow (A + U + U)$ $\quad \Delta F_2$,
$A + A \rightarrow (A + A)$ $\quad \Delta F_A$,
$(A + U + U) \rightarrow A + 2\,U \rightarrow {}^1/_2 (A + A) + 2\,U$,
$\Delta F = -\Delta F_1 - \Delta F_2 + {}^1/_2 \Delta F_A$.

ΔF_1 and ΔF_2 have been determined from spectrophotometric titrations of U, 1 A − 1 U and 1 A − 2 U solutions to alkaline pH values [10]. The ΔF_A values are given (at pH $\gg$ pK) by $\Delta F_A = 2.3\ RT\ (pH - pH_{A+A})$.

At the transition pH we have $\Delta F = 0$: there is compensation between the free energy change $-\Delta F_1$ or $-\Delta F_1 - \Delta F_2$ and the free energy change ${}^1/_2 \Delta F_A$.

ω	pH_0 (±0.2)	pH_{s1} (±0.05)	pH_{s2} (±0.05)
0.01	—	5.12	—
0.02	—	4.51	—
0.03	—	4.35	—
0.04	—	4.07	—
0.08	—	3.71	—
0.1	4.20	3.40	—
0.2	—	—	3.09
0.3	4.40	—	2.82
0.4	—	—	2.70
0.6	4.40	—	2.60
0.8	4.40	—	2.49
1	4.40	—	2.35

Table 1. Transition pH values of 1 A−1 U and 1 A−2 U solutions at 20 °C and several ionic strengths.

From spectrophotometric titration studies to alkaline and acid pH's (tables 1, 2, 3 and 4) we obtained at $\omega = 1$:

ω	pH_{A+A}
1	5.55
0.6	5.64
0.45	5.72
0.3	5.77
0.2	5.84
0.1	5.95
0.05	6.10
0.04	6.14
0.03	6.22
0.02	6.23
0.01	6.22

Table 2. Transition pH values of A at various ionic strengths.

ω	pK uridine	pK poly U	ΔpK	pK adenosine	pK poly A
1	9.11	9.33	0.22	3.70	3.92
0.3	9.18	9.71	0.53	3.60	4.13
0.1	9.27	10.05	0.78	3.57	4.35
0.03	9.32	10.42	1.10	3.53	4.63
0.01	9.38	10.76	1.38	3.52	4.90

Table 3. pK values of A in function of ionic strength derived from pK values of adenosine, uridine and U [21].

ω	pK U	$\Delta pH \cong -\Delta F$	pH_s	pK A
1	9.33	0.87 + 0.59	2.35	3.81
0.3	9.71	0.69 + 0.25	2.82	3.76
0.1	10.05	0.48	3.40	3.88
0.03	10.42	0.24	4.35	4.59
0.01	10.76	0.04	5.12	5.16

Table 4. pK values of A from acid and basic dissociations of (A+U) and (A+U+U).

- ΔpK, representing the free energy change of the dissociation
  $(A + U + U) \rightarrow A + 2\,U$ $\quad \Delta pK = 0.87 + 0.59$
- the acid dissociation pH_s of (A + U + U): $pH_s = 2.35$
- the pH_{A+A} of the interaction $A + A \rightarrow (A + A)$ $\quad pH_{A+A} = 5.55$.

The relations are:

- the free energy change $-\Delta F_1 - \Delta F_2$:

at $pH > pK$ of A:
$$-\Delta F_1 - \Delta F_2 = 2.3\ RT\,(0.87 + 0.59),$$
at $pH < pK$ of A:
$$-\Delta F_1 - \Delta F_2 = 2.3\ RT\,(0.87 + 0.59 + pH - pK)$$

– the free energy change ΔF_A:

at $pH > pK$ of A: $\Delta F_A = 2.3\ RT(pH - 5.55)$,

at $pH < pK$ of A: $\Delta F_A = 2.3\ RT(pK - 5.55)$.

For $\omega = 1$ we find the transition $(A+U+U) \rightarrow {}^1/_2(A+A) + 2\,U$ at pH = 2.35.
There are two possibilities:

1. $pH = 2.35 > pK$ of A,
$-\Delta F_1 - \Delta F_2 = -\Delta F_A$,
$2.3\ RT(0.87 + 0.59) = {}^1/_2\ 2.3\ RT(5.55 - 2.35)$.

The experimental pH transition values are inconsistent with this relation.

2. $pH = 2.35 < pK$ of A,
$2.3\ RT(0.87 + 0.59 + 2.35 - pK)$
$= {}^1/_2\ 2.3\ RT(5.55 - pK)$.

From this we obtain $pK = 2.07$, a value which is conflicting with the supposition $pH = 2.35 < pK$. The assumption that the same free energy change is involved in both the formation of (A + A) from A, set free from (A + U) at lower pH values, and from a pure A solution in the same conditions is therefore contradicted by the experimental transition pH values of alkaline and acid dissociations of A – U complexes. This supposition involves that energetically the configuration of the single stranded A is the same as that of A belonging to (A + U) and must be rejected.

Another supposition would be that when A, set free from (A + U), associates to (A + A) a quantity of free energy ΔF is liberated which is actually a fraction of the free energy change $\Delta F = \delta \cdot \Delta F_A$, set free when (A + A) is formed from a neutral solution of A by lowering the pH. For 1 A – 1 U solutions of higher ionic strengths we have two relations between the ΔF values:

– in the pH range 5.0 to 4.0, transition from the two stranded to the three stranded complex 2 $(A+U) \rightarrow (A+U+U) + A \rightarrow (A+U+U) + {}^1/_2(A+A)$

– at lower pH values, the dissociation of (A + U + U) to the component polynucleotides $(A+U+U) \rightarrow 2\,U + A \rightarrow 2\,U + {}^1/_2(A+A)$.

In both cases we have the transition $A \rightarrow {}^1/_2(A+A)$. From the first relation we can determine the fraction coefficient δ and from the second relation the pK of A.

For the transition 2 $(A+U) \rightarrow (A+U+U) + A \rightarrow (A+U+U) + {}^1/_2(A+A)$

$A + U \rightarrow (A+U)$ ΔF_1,

$(A+U) + U \rightarrow (A+U+U)$ ΔF_2,

$A + A \rightarrow (A+A)$ $\Delta F_A = 2.3\ RT(pH - pH_{A+A})$,

$2(A+U) \rightarrow (A+U+U) + {}^1/_2(A+A)$

$\Delta F = -\Delta F_1 + \Delta F_2 + {}^1/_2\ \Delta F_A$.

At the transition pH_0, we have $\Delta F = 0$ or $-\Delta F_1 + \Delta F_2 = {}^1/_2\ \delta\ 2.3\ RT(pH_{A+A} - pH_0)$, and δ can be calculated (table 5).

ω	$-\Delta F_1 + \Delta F_2$	pH_{A+A}	pH	δ
1	2.3 RT (0.28)	5.55	4.4	0.50
0.6	2.3 RT (0.35)	5.64	4.4	0.55
0.3	2.3 RT (0.44)	5.77	4.4	0.65

Table 5. The free energy reducing factor δ of the interaction A + A → (A + A) determined from the transition of the two- to the three stranded structure.

From this the pK values of A can be calculated. At the melting pH of (A + U + U) to the component polynucleotides we have

$$(A+U+U) \xrightarrow{50\%} A + 2\,U \rightarrow {}^1/_2(A+A) + 2\,U\,.$$

There is compensation between the free energy change of the dissociation $(A+U+U) \rightarrow A + 2\,U$ (at pH < pK this value amounts to $-\Delta F - \Delta F_2 + 2.3\ RT(pH - pK)$) and the free energy change ΔF_A of the interaction $A + A \rightarrow (A+A)$ (at pH < pK) is given by $\Delta F_A = 2.3\ RT(pH_{A+A} - pK)$.
At pH_s:

$$\Delta F = -\Delta F_1 - \Delta F_2 + 2.3\ RT(pH_s - pK) + \Delta F_A = 0\,.$$

Table 6 gives the pK values of A derived from this relation. At lower ionic strengths we have only one relation between the two parameters (δ and the pK of A) so that it is impossible to obtain quantitative estimates of the pK of A from the acid dissociation of 1 A – 1 U solutions. We do get a qualitative estimate of the pK of A when assuming at lower ionic strengths a δ value of the interaction $A + A \rightarrow (A+A)$ almost equal to those determined at higher ionic strengths.

ω	δ	$-\Delta F_1 - \Delta F_2$	pH_s	pK of A
1	0.50	2.3 RT (1.46)	2.35	3.23
0.6	0.55	2.3 RT (1.27)	2.60	3.20
0.3	0.65	2.3 RT (0.94)	2.82	2.90

Table 6. pK values of A from acid dissociation experiments of (A+U+U) aggregates.

When taking at $\omega = 0.1$ a δ value of 0.6 we can calculate the pK of A. At the transition pH_s, there is again compensation between $-\Delta F_1$ of the dissociation $(A+U) \rightarrow A+U$ and the free energy change ΔF_A of $A+A \rightarrow (A+A)$.

When $pH_s < pK$:

$$\Delta F_1 = {}^1/_2\,\Delta F_A \text{ or } 2.3\,RT(0.48 + pH_s - pK) = {}^1/_2\,0.6[2.3\,RT(5.95 - pK)].$$

We thus obtain $pK = 3.0$: this is also inconsistent with the supposition $pH_s = 3.4 <$ pK. The reasonable supposition that the δ values would be about 0.4 or greater would lead to the result that the pK of A is lower than the transition pH_s of $A+U \rightarrow {}^1/_2(A+A)+U$: the pH_s value then is the upper limit of the pK of A.

ω	I	II	III	IV
1	3.92	3.81	—	3.23
0.6	—	—	—	3.20
0.3	4.13	3.76	—	2.90
0.1	4.35	3.88	—	<3.4
0.03	4.63	4.58	—	<4.35
0.01	4.90	5.16	—	<5.1

Table 7. pK values of A in function of ionic strength.

Table 7 gives the pK values of A according to the different suppositions:

I: pK values if the phosphate backbone alone determines the electrostatic potential of A.

II: pK values from alkaline and acid dissociation curves when the acid dissociation of $(A+U)$ is not influenced by the formation of $(A+A)$ from the component A.

III: pK values if the assumption is made that A, set free from $(A+U)$ at lower pH, associates to $(A+A)$ with the same free energy change ΔF_A as that involved in the formation of $(A+A)$ form a neutral solution of A by lowering the pH. With this supposition no pK values could be calculated from the experimentally determined transition pH values.

IV: pK values if we suppose that A from $(A+U)$ associates to $(A+A)$ with the liberation of a quantity of free energy that is a fraction of the free energy change ΔF_A originating from the $(A+A)$ formation in a neutral solution of A by lowering the pH.

The pK values of II and IV are lower than the corresponding values of I: this suggests that the electrostatic potential of A is not only dependent on the phosphate backbone but that other parameters probably influence the potential too. The values of IV are perhaps the most reliable approximations to the real values.

These pK values can be used in studying the energetic and thermodynamic basis of $(A+A)$ interactions. The free energy change of $(A+A)$ formation according to the two step reaction $A+H^{\oplus} \rightarrow AH^{\oplus}$ and $A+AH^{\oplus} \rightarrow (A+AH^{\oplus})$ can be determined from the transition pH_{A+A} and the pK of A.

We proposed for this free energy change

$$\Delta F_A = \Delta F_0 + \Delta F_{prot} \text{ and } \Delta F_0 = -2.3\,RT(pH_{A+A} - pK).$$

The calculated ΔF_0 values can then be compared to the ΔH values of the $(A+A)$ formation determined by RAWITSCHER et al. [25].

A quantitative comparison between the ΔH and ΔF values is impossible as the ΔH and ΔF values do not refer to the same temperatures and ionic strengths. A qualitative comparison, based on $\Delta F = \Delta H - T\,\Delta S$, shows that the entropy change of the interaction $A+A \rightarrow (A+A)$ is small when compared to the entropy changes of $A-U$ interactions.

Several reasons may account for the difference in entropy changes of $A-A$ and $A-U$ interactions:

- for $A-A$ interactions the single stranded A chains are believed to be base-stacked so that the degree of order-disorder is not changed much by $A-A$ interactions.
- the contribution of the solvent can be different for $(A+U)$ where the surface charge density is high (2 negative charges per 3.4 Å) and for $(A+A)$ where we probably deal with a reduced density by interaction between $NH_3^{\oplus}$ and phosphate groups [26].

Ionic strength influences A and $(A+A)$ in a different way than U and $(A+U)$ [27]. The pK decrease of U [21, 22] with increasing ionic strength can be duly accounted for by the screening-off effect on the electrostatic forces of the phosphate backbone so that the electrostatic potential of the macromolecule is reduced and its pK values come nearer to the pK of uridine. The $(A+U)$ is stabilised by an increase

[25] M. A. RAWITCHER, PH. D. ROSS, and J. M. STURTEVANT, J. Amer. chem. Soc. **85**, 1915 [1963].

[26] R. F. STEINER and R. F. BEERS, Polynucleotides, Elsevier Publishing Co., Amsterdam 1961, chapter 7.

[27] D. BARSZCZ and D. SHUGAR, Acta biochim. polon. **11**, 481 [1964].

in ionic strength because the destabilising electrostatic forces between the two polymer chains are screened off.

The influence of ionic strength on the pK of A is made up by several contributions:

1. the electrostatic attraction between the negative phosphate groups and the $NH_3^{\oplus}$ groups will result into higher pK values: an increase in ionic strength will weaken the electrostatic potential and so reduce the pK.
2. the protonated $NH_3^{\oplus}$ groups contribute to the electrostatic potential and reduce the pK: a screening-off at higher ionic strengths will increase the pK.
3. the base stacking in the single stranded A chain is able to influence the electrostatic potential and the pK of A.

The collective contribution of these factors is reflected by a slight pK lowering at increasing the ionic strength (Table 7, IV).

(A + A) is influenced as well by ionic strength:

1. the destabilising electrostatic repulsion of the two polyphosphate chains is screened off at higher ionic strengths: the effect is a higher stability.
2. the electrostatic attraction between $NH_3^{\oplus}$ and phosphate groups is screened off at higher ionic strengths: the effect is a lower stability.

ω	T[°C]	ΔH cal./mole (A + A)	ΔF_0 cal./mole (A + A)
0.1	10	−3.600	
0.1	25	−3.100	
1	20		−3.100
0.6	20		−3.300
0.3	20		−3.900

Table 8. Comparison between the ΔF and ΔH values of the interaction A + A → (A + A).

The summation of the relative contributions of all factors determines the ultimate result. From our results (table 8) in a limited range of ionic strengths ($0.3 < \omega < 1$) we may conclude that a decrease in ionic strength slightly increases (A + A) stability.

An increase in ionic strength results into a displacement of the transition pH to lower pH values; this precludes an interpretation of the stability changes of (A + A) because the pK values at lower ionic strengths are not known. Several authors have studied the thermostability of (A + A) at constant ionic strengths and different pH values [4, 14, 15]. They all found a linear increase of the transition temperature of (A + A) → A + A when the pH of the solution is decreased; the plots for different ionic strengths have the same slope but a higher ionic strength displaces the straight lines to lower pH values; this is in entire agreement with our expression of the free energy change of (A + A) formation, $\Delta F_A(pH) = 2.3\,RT(pH - pH_{A+A})$, and our experimental pH_{A+A} values. The thermostability study of (C + C) in function of pH at constant ionic strength [28] proved that the (C + C) structure is stabilised or destabilised according to the pH of the medium: from pH 5.5 to pH 4.0 we have the proton stabilised zone that may be described by our expression of the free energy change $\Delta F(pH) = 2.3\,RT(pH - pH_{trans})$. At pH 3.5 and lower, we have the destabilised zone: at that point the pH is probably below the pK of C and the protonation of the second C chain destabilises the (C + C) aggregates. This involves that the pK of C is to be situated between pH 4.0 and 3.5: from the pK values of cytidine [21] we would expect a pK value of 4.5 (±0.2): for C also the pK is thus markedly lower than the pK we can foresee from that of cytidine. The study of the thermostability of (A + A) aggregates in function of pH shows no deviation from a linear relationship between the transition temperature and the pH, indicating that at pH 4.0 we have not yet obtained the zone of proton destabilisation: this agrees with a pK of A lower than 4.0 and confirms that the pK values of column I of table 7 overestimate the real pK values of A.

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[28] E. O. AKINRIMISI, C. SANDER, and P. O. P. Ts'o, Biochemistry **2**, 340 [1963].